# Micropropagation and in vitro grafting techniques to assist the selection of a pistachio rootstock from a population of terebinth (*Pistacia terebinthus* L.) collected in the SE of Spain

E. Garcia[1]; I. Imbroda[2]; P. Lorente[1]; J.A. Marín[1]; A. Arbeloa[1]; I.M.G. Padilla[2]; A. Barceló[2]; P. Andreu[1]

[1]Estación Experimental de Aula Dei-CSIC. Apartado 13034, 50080 Zaragoza, Spain
[2]IFAPA, Cortijo de la Cruz s/n, Churriana, 29140 Málaga, Spain

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## Abstract

**Pistachio culture is hampered both by the difficulty of propagation by grafting varieties of interest and by the lack of a clonal selected rootstock. The application of in vitro culture techniques would allow the clonal propagation of *Pistacia* species, as well as to perform in-depth studies of factors affecting grafting. In vitro *P. vera* cultures were initiated from nodal explants, whereas cultures of *P. terebinthus* were initiated from in vitro-germinated seeds. The multiplication rate of both species in vitro was close to 2 shoots per shoot every 3 weeks. A high rooting percentage (82%) of *P. terebinthus* was obtained in a modified WPM medium and plants were successfully transplanted to the greenhouse. Shoot apices of *P. vera* of 10-20 mm length were grafted in vitro on rooted or unrooted shoots of *P. terebinthus* in which the apex was removed, and the evolution of the graft unions was studied. Over 70% of the grafted plants survived during the first 3-5 weeks and some plants continued their growth in the greenhouse. Similarly, control self-grafted *P. terebinthus* were studied, and these homografts showed that while the technique of grafting in vitro is promising in studying graft evolution, it needs to be improved by identifying the factors that affect cell necrosis.**

## INTRODUCTION

Pistachio culture is becoming highly interesting in Spain, where traditional crops as grape and olive have reduced their profitability. However, its expansion is hampered both by the difficulty of grafting varieties of interest on selected rootstocks, and by the lack of a selected clonal rootstock. Terebinth (*Pistacia terebinthus* L.), as a native plant, has an adequate adaptation to Spanish climate and soil, as well as moderate vigor and tolerance to soil diseases making it very interesting as pistachio rootstock (Guerrero et al., 2004). However, terebinth rootstock selection is first based on seed propagation, since it does not propagate by cuttings (Guerrero et al., 2002; Gijón et al., 2010). Lack of an adequate method of propagation is though limiting clonal selection of this rootstock

We have faced up these difficulties in two complementary ways: 1) collecting well adapted autochthonous clones of *Pistacia terebinthus* in the wild, and selecting among the collected plants for vigor and grafting competence; and 2) applying in vitro culture techniques for the clonal propagation of *Pistacia* species, as well as for studying factors affecting grafting.

In this work, a preliminary study applying in vitro culture techniques for the clonal propagation of *Pistacia* species has been developed as a tool for rootstock selection. *P. terebinthus* and *P. vera* have been micropropagated from both juvenile (Chelli y Drira, 2002; Benmahioul et al., 2009; García et al., 2010) and adult explants

(Gannoun et al., 1995; Onay, 2000; Tilkat el at., 2009; García et al., 2010), frequently showing in vitro adaptation problems like the abundant presence of exudates in the culture medium (Tabiyeh et al., 2006). Micropropagation protocols should be adjusted to our plant material and culture conditions to improve, not only micropropagation rates, but also shoot quality in both species.

Pistachio presents numerous grafting failures. Guerrero et al. (2004) showed that pistachio grafting success depends on numerous factors: temperature, humidity, oxygenation, rootstock activity or rootstock diameter. All these factors lead to a frequently low graft success since environmental factors are beyond our control. Unlike other fruit trees where the problems are focused on graft compatibility, pistachio grafting seems to be influenced by the physiological state of rootstock and scion. Thus, initial grafting phases should be carefully understood to discriminate different problems and bottlenecks concerning graft evolution. Thus, we propose a different approach to study pistachio grafting based on in vitro techniques, which provide a suitable and flexible method, easier to control and to study. We carried out the in vitro propagation of *P. terebinthus* and *P. vera* and thereafter their grafting in vitro. Micrografting has been applied in pistachio (Onay et al., 2004) and other fruit trees species (Jonard, 1983) as a method to rejuvenate (Barghchi, 1985; Padilla and Encina, 2011) or to produce disease free scions (Deogratias et al., 1986).

In this work we describe 1) preliminary results of the selection of *P. terebinthus* attending to vigour and seed germination capability, and 2) the use of in vitro grafting as a tool that allows us to study the initial phases of graft evolution, even at the histological level. Besides, the possibility of making homografts from *P. terebinthus* gives us the opportunity to have a control treatment to differentiate technical problems from the lack of graft affinity

## MATERIALS AND METHODS

### *P. terebinthus*

A collection expedition of *P. terebinthus* was performed in the mountain ranges of Jaen and Granada, in the SE of Spain, looking for both seed germination and plant vigour. Collected mature seeds were washed and dried, counted and weighted and cold stored at 4-5ºC. Previously to the sowing date, in March, half of the seeds from each tree were treated with 2% Ethrel (Nufarm) for 24 h at 4-5ºC. Seeds were sowed in forestall cells containing a mixture of peat: coconut fiber: perlite and maintained in a climate greenhouse. Germination data were recorded every two weeks and length and number of nodes were recorded at 8 weeks**.**

**S**hoot tips of *P. terebinthus* seedlings were clonally multiplied in vitro. Three different media were tested: modified DKW (Driver and Kuniyuki, 1984), MS (Murashige and Skoog, 1962) and WP (Lloyd and McCown, 1980), all media were supplemented with 0.5 µM IBA, 5 µM BA, 30 g $L^{-1}$ sucrose, and 7 g $L^{-1}$ agar (Bacto-agar, Difco, Fisher Scientific). Shoots were subcultured every three weeks and culture media were compared attending to: apical necrosis (%), average shoot length (mm), number of internodes per shoot and multiplication rate (number of shoots per shoot). The whole experiment was repeated two times at different dates with 56 shoots per repetition.

Shoots of *P. terebinthus* were rooted on two different media: modified MS and WP, both with half-strength macronutrients, 5 µM IBA and without BAP in the culture chamber. Rooting percentage in WP (78 shoots) or MS (24 shoots) was recorded after 4-8 weeks.

***P. vera***

Simultaneously one-node explants of *P. vera* plants grown in the field were taken in spring and disinfected with 0.05% $HgCl_2$. Explants (33 one-node explants) were cultured in modified DKW medium (Driver and Kuniyuki, 1984) with ascorbic acid (0.01g $L^{-1}$) for two months in the culture chamber at 22 ºC under a 16 h photoperiod with cool-white fluorescent tubes (35 µmol $m^{-2}$ $s^{-1}$). New shoots were transferred onto 30 ml fresh medium for multiplication. Three different media were also tested like in *P. terebinthus* and shoot growth analyzed in a similar way.

**In vitro grafting**

In vitro *P. vera* shoot tips (1-2 cm long) were grafted in vitro on rooted or unrooted shoots of *P. terebinthus* (4 cm long) with their apex cut off. Scions with the base cut in v-shape were fitted onto vertical slits made on the decapitated rootstocks (Onay et al., 2004; Thimmappaiah et al., 2002). A drop of agarose 4% was added (Jonard et al., 1983) to the graft union. Grafting was done on aseptic conditions under a stereoscopic microscope. Homografts of *P. terebinthus* were also made as controls. A total of 66 grafts were done in vitro 50 of which belonged to *P. vera*/*P. terebinthus* combination and 16 to P. *terebinthus/P. terebinthus.* Grafted shoots were cultured in vitro in the multiplication medium for 1-2 months and thereafter were potted in a mixture of peat and perlite (1:1) and acclimatized in the greenhouse.

Histological studies were undertaken after 4-8 weeks following grafting. Shoots were sectioned under a stereoscopic microscope (31 shoots for *P. vera*/*P. terebinthus* and 5 shoots for P. *terebinthus/P. terebinthus*). Longitudinal handmade cuts were observed in a fluorescence microscope (Olympus AX60) and pictures were taken with an Olympus C7070WZ camera. Cell proliferation and differentiation were observed at the grafting interface and vascular connections between rootstock and scion were recorded following 0.01% acridine orange stain (Pearse, 1968).

## RESULTS AND DISCUSSION

***P. terebinthus***

Six terebinth trees were selected from four different locations and their seeds were collected to study their germination rate and seedling vigor.

Higher germination percentages were obtained after 4 to 6 weeks, while not significant increases were obtained later. Except in PT10, germination percentages were higher in Ethrel treated seeds, however, considerable variability among trees was found (Fig. 1) ranging from 27% (PT8) to 80% (PT6).

A great variability was also observed on seedlings development among the tested trees and the Ethrel treatment had a significant positive effect on seedlings length and nodes number (Fig. 2). These results agree with those from Guerrero et al. (2002), who observed significant differences among *P. terebinthus* trees from autochthonous populations, with remarkable vigor differences among individuals.

Multiplication rate was close to 2 every three weeks (Table 1) in the three tested media, although DKW medium showed statistically higher shoot quality: longer shoots, higher number of internodes and less apical necrosis than MS or WP. In previous works Chelli and Drira (2002) found better results with MS medium compared to OM medium (Rugini, 1984). Here DKW showed to be a very adequate medium for this material. Rooting was achieved in modified WP medium in 82% of the shoots (Figure 3A) but decreased to 42% in modified MS medium. Root development was gradual from week 4 to week 8 after rooting treatment, and plants were grown successfully in the greenhouse.

***P. vera***

Initial nodal explants showed a 40% survival after 12 weeks in culture. Exudates were controlled with the addition of ascorbic acid to culture media during the first subcultures. One single bud was selected for posterior culture. Multiplication rate in the three tested media varied between 2.1 and 3.3 every 3 weeks (Figure 3B). WP medium showed statistically higher multiplication rates, although new shoots showed higher apical necrosis, thus DKW medium, like in *P. terebinthus*, was considered the most appropriate medium for *P. vera* multiplication since shoots were longer, with higher number of internodes and lower apical necrosis. Other authors, however, used a modified MS medium for *P. vera* micropropagation (Onay, 2000; Tilkat et al., 2009).

**Grafting**

Grafting survival after 3-5 weeks was 73% in homografts and 44% in *P. vera/P. terebinthus* grafts and some plants continued their growth in the greenhouse.

*P. vera*/*P. terebinthus* grafts showed variable aspect from healthy (18%) to unhealthy with darkened tissues (49%). While some grafts showed thickened unions (26%), others did not adhered at all (7%). However, histological examination showed that 80% of the grafts displayed partial contact between rootstock and scion and 67% showed differentiated xylem cells in this area (Table 2, Figure 3C,D). Although most grafts showed a positive evolution with callus cell development, all the studied grafts presented necrotic areas that indicate discontinuity zones. It is possible that graft evolution in later phases could form vascular connections besides necrotic areas that eventually could lead to graft success.

On the other hand, most of homografts showed healthy unions (73%) (Figure 3E). Histological examination, 8 weeks after grafting, showed cell proliferation and xylem cell differentiation in all the studied grafts (Table 2, Figure 3F). However, the number of grafts with partial contact was 80%, whereas 60% showed necrotic zones. These results suggest the need of technical improvements in grafting in order to ensure physical proximity that promoted vascular development that guarantee the supply of water and nutrients to the variety. The presence of cell necrosis in homografts, where tissue affinity is not the problem, suggests that an improved mechanical fixing system should be tested besides agarose. In this sense, Jonard et al. (1983) added agar to improve bud nutrition and Gebhart et al. (1988) used silicon bands to fix grafts. Although mechanical union should be improved at initial phases, *P. vera*/*P. terebinthus* plants growing in the greenhouse were able to develop vascular connections between rootstock and scion.

Related symptoms of grafting failure, as a small contact between partners or the presence of necrotic cells can help us to understand irregular grafting success in the field and to determine the factors that apply. In vitro grafting has allowed us to observe initial grafting events as cell proliferation and differentiation in a short period of time that can be related to grafting success, and hence, becoming early indicators of success (Garcia et al., 2010).

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## Tables

Table 1. Comparison of different culture media in the multiplication phase of *Pistacia vera* and *P. terebinthus*. Mean values (± s.e.m.) of shoot length, number of internodes, multiplication rate (number of shoots per shoot) and the percentage of apical necrosis (*,***,n.s.: P<0.05, P<0.001, not significant, respectively; n: sample size)

| | n | Length (mm) | Intermodal number | Multiplication rate | Apical necrosis(%) |
|---|---|---|---|---|---|
| *P. vera* | | | | | |
| DKW | 111 | 23.68 ± 1.00a | 6.3 ± 0.20a | 2.5 ± 0.11b | 25.22c |
| MS | 112 | 17.46 ± 0.61c | 4.9 ± 0.15b | 2.1 ± 0.09c | 47.32b |
| WP | 112 | 20.64 ± 0.75b | 5.1 ± 0.16b | 3.5 ± 0.17a | 61.61a |
| signification | | *** | *** | *** | *** |
| *P. terebinthus* | | | | | |
| DKW | 30 | 30.9 ± 3.25a | 6.1 ± 0.36a | 1.8 ± 0.13 | 10.0b |
| MS | 30 | 19.8 ± 1.45b | 4.9 ± 0.28b | 1.8 ± 0.19 | 36.7a |
| WP | 30 | 20.5 ± 1.48b | 4.1 ± 0.28b | 1.9 ± 0.15 | 26.7ab |
| signification | | *** | *** | n.s. | * |

Table 2. Histological study of grafts. Percentage of in vitro-grafts *P. vera/P. terebinthus and P. terebinthus/P. terebinthus* showing at the interface cellular proliferation, xylem cell differentiation, partial contact between partners and cell necrosis.

| | n | Cell Proliferation | Cell Differentiation | Partial Contact | Cell Necrosis |
|---|---|---|---|---|---|
| *P. vera/P.terebinthus* | 31 | 90 % | 67% | 80% | 100% |
| *P.terebinthus/P.terebinthus* | 5 | 100% | 100% | 80% | 60% |

## Figures

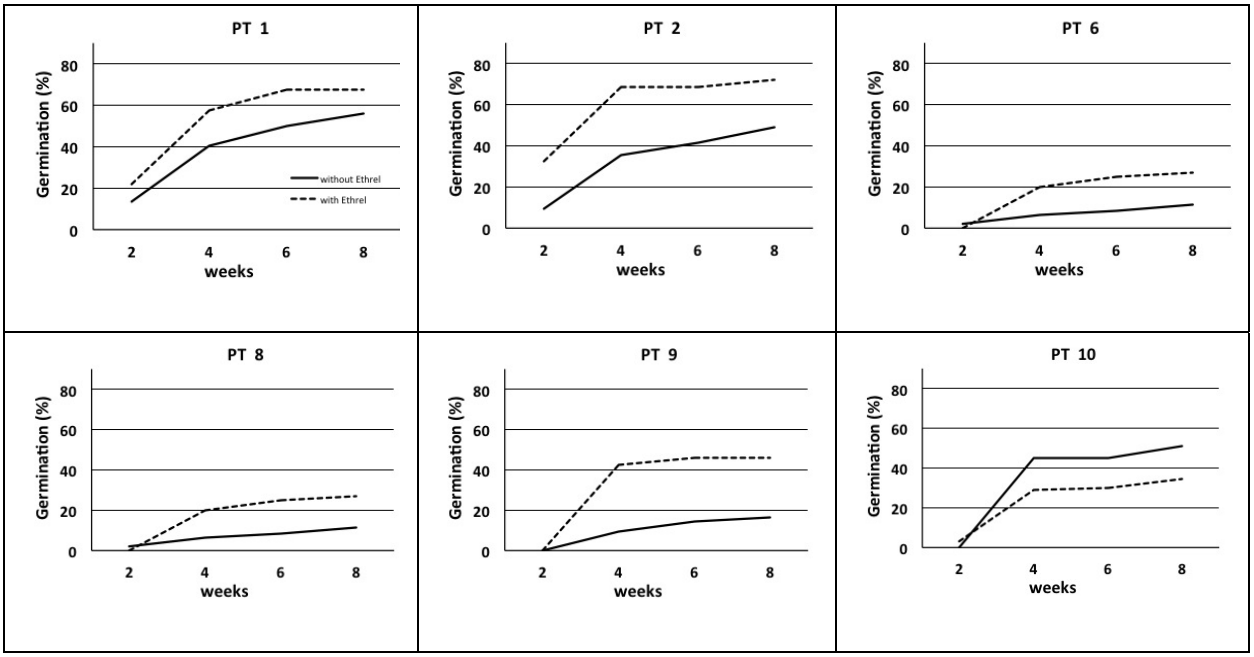


Figure 1. Effect of Ethrel on germination percentage of *P. terebinthus* seeds.

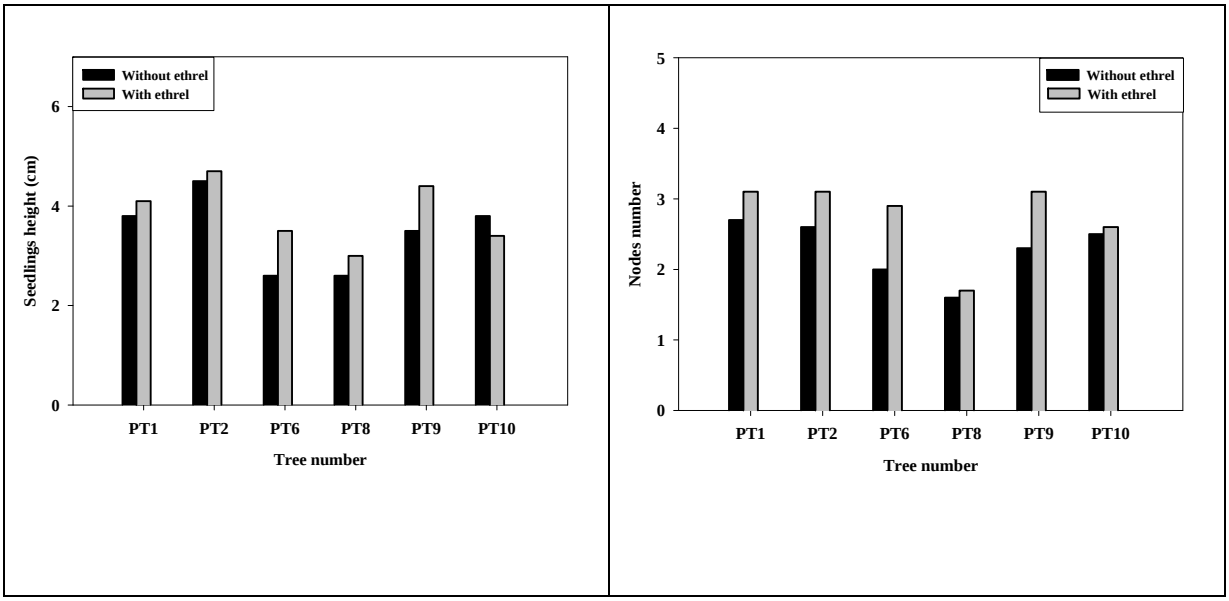


Figure 2. Effect of Ethrel on *P. terebinthus* seedlings height (left) and number of nodes (right) after 8 weeks.

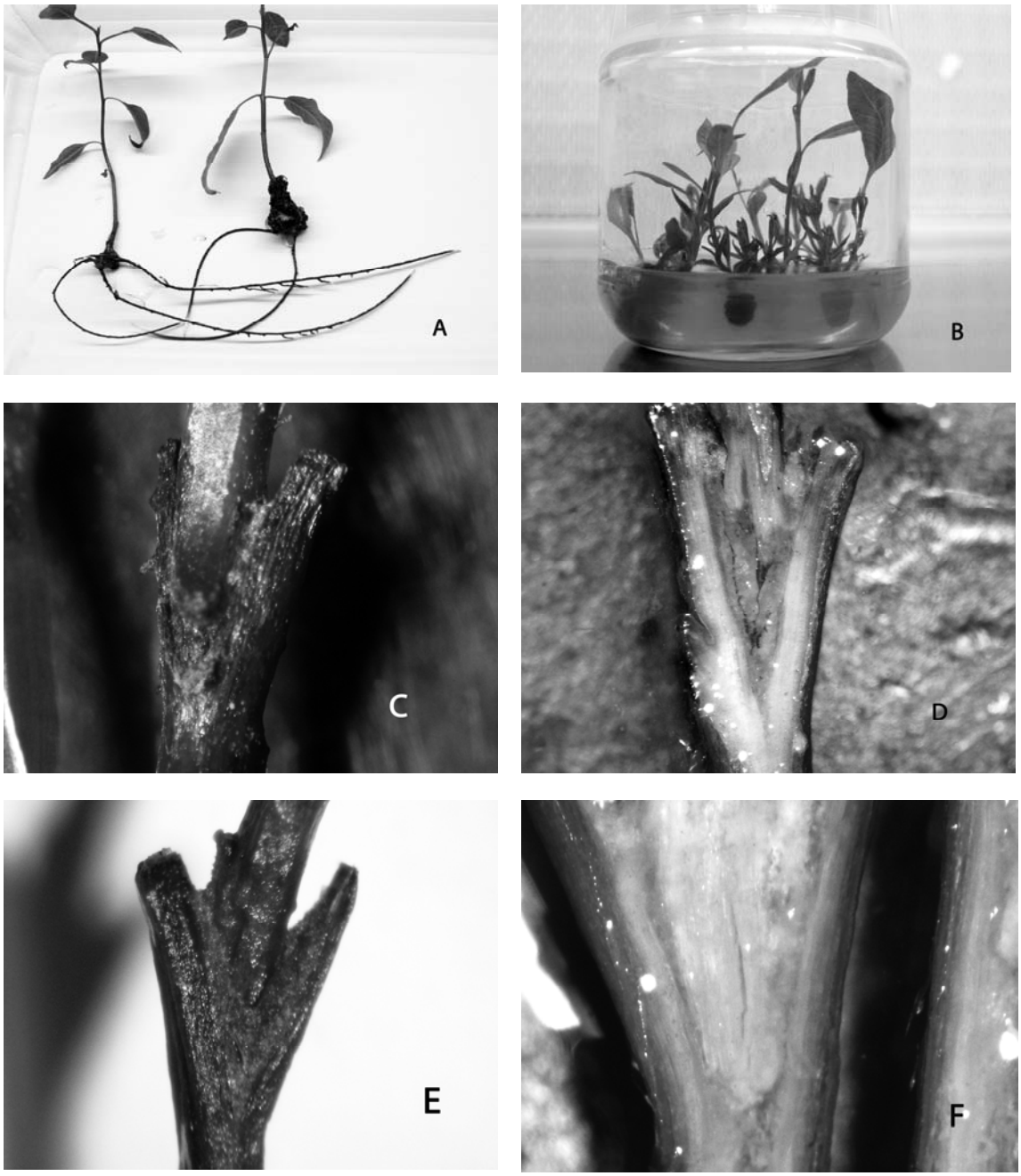


Figure 3. A) *Pistacia terebinthus* shoots rooted in vitro. B) *P.vera* shoots in multiplication medium. C) *P. vera/P. terebinthus* graft. D) Longitudinal section of *P. vera/P. terebinthus* graft. E) *P. terebinthus/P. terebinthus* homograft. F) Longitudinal section of *P. terebinthus* homograft